other functions, transactivates the AAV promoters present on the helper plasmid that direct the transcription and translation of AAV rep and cap regions. Upon subsequent culture of the host cells, rAAV virions (harboring the nucleotide sequence of interest) and helper virus particles are produced.

[0012] Adeno-associated virus (AAV) has a stable capsid that is composed of 60 copies of three capsid proteins (VP1, 2 and 3). (Berns K, and Parrish C R, Parvoviridae. in Fields Virology, 5th ed. Ed. by David M. Knipe, Wolters Kluwer/Lippincott Williams & Wilkins, Philadelphia (2007). The commonly used recombinant AAV ("rAAV") vector serotypes are AAV1-9, each with different tissue tropisms. (Zincarelli C, et al., Analysis of AAV serotypes 1-9 mediated gene expression and tropism in mice after systemic injection, Molecular Therapy 16:1073-1080 (2008)).

[0013] Recombinant AAV is one of the most promising viral gene transfer vectors because it has high gene transfer efficiency, long-term gene expression, natural replication deficiency, and is non-pathogenic. (Coura R S and Nardi N B., The state of the art of adeno-associated virus-based vectors in gene therapy, Virology J. 4:99-105 (2007)). One of the major challenges for using rAAV vectors has been the difficulty in large scale production of vectors for preclinical target identification/validation studies, or use in large animal models and clinical trials of human gene therapy. (Allay, J A et al., Good manufacturing practice production of self-complementary serotype 8 adeno-associated viral vector for a hemophilia B clinical trial, Hum Gene Ther. 2011; 22:595-604 (2011)). The principle of rAAV vector production is to supply three components to cultured cells: the gene of interest ("GOI") expression cassette flanked by inverted terminal repeats ("ITR"s) of AAV, the rep and cap genes, and trans-acting helper functions. Triple transfection of adherent HEK 293 cells is a commonly used method for rAAV vector production (Xiao, X. and Samulski, R J., Production of high-titer recombinant adeno-associated virus vector in the absence of helper adenovirus, J. Virol. 72:2224-2232 (1998)) and is reported to be efficient (Lock, M. et al, Rapid, simple, and versatile manufacturing of recombinant adeno-associated viral vectors at scale, Hum. Gene Ther. 21:1259-71 (2010)). However, cell culture work involved in rAAV production including expansion, seeding and transfection of adherent HEK 293 cells is cumbersome and resource intensive. Therefore, using cells suspended in aqueous liquid medium ("suspension cells") for rAAV vector production is desirable due to its scalability and cost effectiveness.

[0014] Several systems using suspension cells to produce rAAV vectors have been developed and described in the literature:

[0015] 1. Insect cell (Sf9 or H5 cells)/baculovirus: This system involves infection of insect cells with two recombinant baculoviruses to provide rep, cap and GOI flanked with ITR and has high production efficiency. (Urabe, M. et al., Insect cells as a factory to produce adeno-associated virus type 2 vectors, Hum. Gene Ther. 13:1925-1943 (2002)). Although insect packaging cell lines were recently developed and the number of required baculoviruses was reduced to one, this system still faces several drawbacks, such as long lead time and genomic instability of the baculovirus. (Aslanidi, G. et al., An inducible system for high efficient production of recombinant production of recombinant adeno-associated virus (rAAV) vectors in insect Sf9 cells, Proc. Natl Acad. Sci. USA 106:5059-5064 (2009)).

[0016] 2. HeLa based cell lines/Ad5 or HSV-1: This approach requires generating a stable rAAV packaging cell line and providing helper functions and transgene cassette using Ad5, or generating a producer cell line and providing helper functions with Ad5 or HSV1. (Gao, G P et al., High-titer adeno-associated viral vectors from a Rep/Cap cell line and hybrid shuttle virus, Hum. Gene Ther. 9:2353-62 (1998); Thorne, B A et al., Manufacturing recombinant adeno-associated viral vectors from producer cell clones, Hum. Gene Ther. 20:707-14 (2009); Toublanc, E. et al., Identification of a replication-defective herpes simplex virus for recombinant adeno-associated virus type 2 (rAAV2) particle assembly using stable producer cell lines, J. Gene Med. 6:555-64 (2004)). The method is suitable for large scale production, but generating stable cell lines is cumbersome and lengthy.

[0017] 3. BHK21 cells/HSV system: This system utilizes two rHSV-1 vectors to deliver cis and trans factors required for rAAV vector production and has been used for large scale rAAV vector production. (Booth, M J et al., Transfection-free and scalable recombinant AAV vector production using HSV/AAV hybrids, Gene Ther. 11:829-37 (2004)). However, the lengthy time to produce the master viral banks, fragility and pathogenicity/immunogenicity of HSV make this method less favorable.

[0018] All of the above methods 1-3 need to ensure the elimination of the virus used from the final rAAV vector preparation.

[0019] 4. Suspension adapted HEK 293 cells/triple transfection: This is a traditional triple transfection method for rAAV vector production, but in suspension-adapted cells instead of adherent cells. Advantages of this method are scalability, flexibility, simplicity and speed, which are important when different combinations of serotypes and/or GOIs and strict timelines are necessary.

[0020] Only a few protocols using suspension-adapted HEK 293 cells/triple transfection have been reported and all were optimized using the one factor at a time (OFAT) method. Park, et al. first explored the possibility of combining suspension HEK 293 cells and polyethyleneimine (PEI) transfection for rAAV2 vector production. (Park, J Y et al., Scalable production adeno-associated virus type 2 vectors via suspension transfection, Biotech. Bioeng. 94:416-430 (2006)). The authors demonstrated that a similar amount of rAAV2 could be generated in suspension cells as compared to adherent cells, and HEK 293T cells were more efficient for rAAV vector production than HEK 293 cells. The cell density (0.5×10^6 cells/ml) and the plasmid ratio (1:1:1; pHelper:pTrans:pCis, an equimolar ratio) were not optimized in the study, but the total amount of DNA was optimized to 3 μg/ml. In addition, media changes before and after transfections were required in this protocol.

[0021] A more comprehensive study by Durocher, et al. optimized the ratio of the three plasmids (1:1:1 in HEK293E cells), the cell density (0.5×10^6 cells/ml, tested densities: 0.5, 1.0 and 2.0×10^6 cells/ml in HEK293F cells), and harvest time (48 h, to obtain higher infectious virus particles ("IVP") in 293F cells), while the amount of DNA (1 μg/ml) and polyethyleneimine (PEI):DNA (2:1) ratio were kept constant. (Durocher, Y et al., Scalable serum-free production of recombinant adeno-associated virus type 2 by transfection of 293 suspension cells, J. Virol. Methods. 144:32-40 (2007)).

[0022] Hildinger, et al. described a more complicated method for rAAV2 production in HEK293E cells. Several